PTO 05-1461

Japanese Patent
Document No. H01-257492

METHOD FOR FORTIFYING THE GENERATION OF HETEROLOGOUS PROTEINS

[Ishu Tampakushitsu no Seisan Zokyo Hoho]

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UNITED STATES PATENT AND TRADEMARK OFFICE
Washington, D.C. January 2005

Translated by: Schreiber Translations, Inc.

Country	:	Japan
Document No.	:	H01-257492
Document Type	:	Kokai
Language	:	Japanese
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Applicant	:	Midori Juji Co., Ltd.
IPC	:	C 12 P 21/00 C 12 N 15/00 //(C 12 N 15/00 C 12 R 1:91)
Application Date	:	March 5, 1987
Publication Date	:	October 13, 1989
Foreign Language Title	:	Ishu Tampakushitsu no Seisan Zokyo Hoho
English Title	:	METHOD FOR FORTIFYING THE GENERATION OF HETEROLOGOUS PROTEINS

Specification

1. Title of the invention

Method for fortifying the generation of heterologous proteins

2. Patent Claims

1. A method for fortifying the generation of heterologous proteins characterized, in a case where heterologous proteins are generated by incubating an animal cell transformed by using a vector into which a DNA sequence capable of coding heterologous proteins has been incorporated, by the addition, to a medium, of butyric acid or a salt thereof.

2. A method specified in Claim 1 wherein said animal cell is CHO-K_1.

3. A method specified in Claim 1 wherein said vector includes an SV 40 initial promotor.

3. Detailed explanation of the invention

(i): Fields of utility

The present invention concerns a method for incubating a reconstituted animal cell. More specifically, it concerns a method for fortifying the generation of heterologous proteins in a case where a reconstituted animal cell is incubated.

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[1] Numbers in the margin indicate pagination in the foreign text.

(ii): Prior art

In early days, *Escherichia coli* was studied mainly as a host for generating targeted heterologous proteins by means of gene reconstituting technologies, and furthermore, bacteria such as yeasts, *Bacillus subtilis*, etc. were frequently used due not only to their incubation friendlinesses but also to advancements of studies on host-vector systems, etc. [refer to Goeddel, D. V., Itakura, K., et al., *Proc. Nath. Acad. Sci. U.S.A.*, 76, 106 (1979) and Nagata, S., Taira, S. H., and Weissmann, C., *Nature*, 284, 1316 (1980)].

In recent years, however, focuses have been shifting to expression systems that use animal cells as hosts for generating substances based on technologies whereby genes of polymer saccharide proteins are reconstituted not only in forms analogous to natural types but also in soluble forms.

Previously known animal cells are instantiated by

CHO-K_1 (Chinese hamster ovary cell; ATCC CCL61),

BHK (newborn hamster kidney cell; ATCC CCL10),

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COS-7 (CV-1 Origin, SV 40 cell; ATCC CRL1651),

Vero (African velvet green monkey kidney cell; ATCC CCL-81),

Etc.

(iii): Problems to be solved by the invention

The next issue to be addressed in this context is how to fortify the generation of heterologous proteins.

At this time, the present inventors have completed the present invention upon the discovery that the generation of heterologous proteins can be fortified by adding, to a medium, butyric acid or a salt thereof in the course of the incubation of a reconstituted animal cell.

(iv): Mechanism for solving the problems

The present invention concerns a method for fortifying the generation of heterologous proteins characterized, in a case where heterologous proteins are generated by incubating an animal cell transformed by using a vector into which a DNA sequence capable of coding heterologous proteins has been incorporated, by the addition, to a medium, of butyric acid or a salt thereof.

(1): Preparation of a reconstituted animal cell

A reconstituted animal cell can be obtained by introducing, into an animal cell selected as a host, a vector into which a DNA sequence capable of coding targeted heterologous proteins has been incorporated based on a gene reconstitution technology for transforming said cell.

There are no special restrictions on the heterologous proteins so long as they are polymer proteins or polymer saccharide proteins.

Such heterologous proteins can be instantiated by urokinase (UK), prourokinase (ProUK), tissue plasminogen activator (t-PA), B-type hepatitic surface antigen (HBsAg), HBsAg inclusive of a

pre-S region (PreS-HBsAg), interferon-γ (IFN-γ), colony forming stimulant factor (CSF), etc.

Moreover, it is also possible to use their active fragments (domains) or derivatives.

DNA sequences capable of coding these physiologically active substances as well as their preparation methods are disclosed in the following literature, etc.:

ProUK: Japanese Patent Application Publication Kokai No. Sho 60[1985]-180591;

ProUK domain: Japanese Patent Application Tokugan No. Sho 61[1986]-156936;

t-PA: Japanese Patent Application Publication Kokai No. Sho 59[1984]-183693 & same Sho 59[1984]-42321;

HBsAg: Same Sho 59[1984]-36698; PreS-HBsAg: Same Sho 56[1981]-63995;

IFN-γ: Same Sho 61[1986]-108397; CSF: Same Sho 61[1986]-199787.

A vector for expressing heterologous proteins may, for example, be constituted, as expressive genes, by an enhancer derived from SV-40, initial region promotor, polyadenylation signal, and genes for coding useful heterologous proteins. Such expressive vectors are instantiated by pSV-G_1 (Japanese Patent Application Publication Kokai No. Sho 61[1986]-177987), pSV-G_2 (Japanese Patent Application Tokugan No. Sho 61[1986]-79086), etc. It is also possible to use promotors derived from other animal viruses and animal cells.

Animal cells are instantiated by CHO-K_1, BHK, COS-7, Vero, human kidney-derived cellular strains (refer to Japanese Patent Application Tokugan No. Sho 60[1985]-290325), Chan hepatic cell (ATCC No. CCL13), chimpanzee hepatic cell (ATCC No. CCL6311), etc. Of these, CHO-K_1 is especially desirable.

The goal of transformation can be achieved by inserting such a vector for expressing heterologous proteins into the nucleus of said host cell.

Methods for introducing reconstituted DNAs to cells are instantiated by the calcium phosphate precipitation method (Strain, et al., *Biochem. J.*, 218, 475 ~ 482, 1984), etc. According to this method, a calcium chloride solution is added dropwise to a phosphate buffer inclusive of reconstituted DNA for forming a microscopic precipitate of DNA calcium phosphate, and said microscopic precipitate is adsorbed onto and taken into the cell.

The transformation is executed by preparing a host cell within a laboratory dish at a ratio of approximately 1 ~ 10 x 10^5 cells 10 mL/100 mm dish and by adding, in terms of DNA content, 2 ~ 4 μg of the vector for expressing heterologous proteins. After the DNA has been taken into the host cell, incubation is carried out, followed by the selection of a transformed cell.

In the context of selecting a cell in which exogenous genes are being expressed, a method wherein chemical-resistant genes are simultaneously introduced for selecting chemical-resistant strains

is used (Splamani, et al., *Ara. Biochem.*, 135, 1 ~ 15, 1983).

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Aminoglycoside 3'-phophotransferase II, which is a neomycin-resistant gene bearing resistance to G-418 within an animal cell, may, for example, be used. Chemical-resistant genes are introduced by means either of direct insertions into the above-mentioned expressing vector or of co-transfection. Usable neomycin-resistant gene expressive plasmids are instantiated by the promotor of SV 40, splicing junction, pSV-G_1-Neo or pSV-G_2-Neo obtained by connecting neomycin-resistant genes to vectors in possession of poly A-added signals, etc.

(2): Preliminary incubation

A reconstituted animal cell is preliminarily proliferated and intergenerationally incubated.

Usable media are instantiated by a Waymouth medium, Dulbecco's modified Eagle medium, F-12 medium, RITC-80-7 medium [*J. Cell. Physiol.*, 111, 155 ~ 162 (1982)], etc., and of these, the F-12 medium and RITC-80-7 are preferred.

It is more desirable, furthermore, to add approximately 10 ~ 20 v/v% of bovine fetal serum.

Moreover, 10 ~ 100 μg/mL of proline may also be added.

After reconstituted animal cells have been implanted into a medium at a density of 10,000 ~ 200,000 cells/mL, they are continuously incubated for 2 ~ 3 days and then, after media have,

if necessary, been exchanged, incubated intergenerationally until the cell count reaches 100,000 ~ 2,000,000 cells/mL.

(3): Main incubation

Usable matrix media are instantiated by a Waymouth medium, Dulbecco's modified Eagle medium, F-12 medium, RITC-80-7 medium, etc., and of these, the F-12 medium and RITC-80-7 are preferred.

It is desirable for the present process to be implemented within a serum-free medium.

It is then that butyric acid or a salt thereof (e.g., sodium salt, potassium salt, etc.), which peculiarly characterizes the present invention, is added.

Its addition ratio is designated within a range of approximately 0.1 ~ 10 mM, preferably 1 ~ 5 mM.

It is also possible to add, to the medium, hormones such as lactoalbumin hydrolyzate, transferrin, various amino acids, various fatty acids, insulin, etc.

It is desirable to introduce, into said medium, air (air: 95%; CO_2: 5%) adventitiously (flow rate: 10 ~ 500 mL/min.), and it is desirable for the temperature to be designated within a range of 20 ~ 37°C. The incubation liquids are exchanged every 2 ~ 3 days or so.

It is thus that heterologous proteins become generated within the medium or cytoplasm.

The generated product may, for example, be recovered from the medium by adventitiously combining such mechanisms as centrifugal

separation, reduced pressure enrichment, salting out fractionalization, gel filtration, enrichment, ion exchange chromatography, affinity chromatography, etc.

In a case where the same is recovered from the cytoplasm, the cells are broken either mechanically or by using a surfactant, etc., and the obtained cell extract liquid is treated according to procedures similar to those for the aforementioned incubated supernatant.

(v): Effects

The method of the present invention is capable of fortifying the generation of heterologous proteins within reconstituted animal cells by 2 ~ 3 times or so.

The method of the present invention is therefore being considered to be an extremely useful method for mass-producing heterologous proteins by using animal cells in the field of genetic engineering.

(vi): Application examples and experimental examples

The present invention will be explained in further detail with reference to application examples and experimental examples, although the present invention is in no way limited to these examples.

Application Example 1

200,000 cells each of the ProUK generative transformed strains obtained in Reference Example 1 (C-68-53 & C-68-61) and CHO-K_1 cells were implanted into 4 mL of an F-12 medium inclusive of 10 v/v% of bovine fetal serum (60 mm dish) and then incubated at 37°C for 3 days. After the medium had then been removed, the incubating dish was washed with 4 mL of a maintenance medium (F-12 or RITC-80-7), and after an additional maintenance medium or a maintenance medium inclusive of 5 mM sodium butyrate had then been added, the cells were incubated at 37°C for 24 hours. The UK activity of the incubation supernatant was measured based on the fibrin flat panel method.

Table I

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Medium	Sodium butyrate	UK generation (U/mL·day)		
		C-68-53	C-68-61	CHO-K_1
F-12	0 mM	49±21 (9)	104±39 (7)	0.49±0.13 (3)
F-12	5	132±52 (9)	331±92 (7)	0.65±0.17 (3)
RITC-80-7	0	91±37 (8)	219±65 (7)	-
RITC-80-7	5	194±72 (8)	485±197 (7)	-

The display numerical values are mean values ± standard deviations.

The numerical values within ( ) are Experimental Example Nos.

Application Example 2

The PreS-HBsAg generative transformed strains obtained in Reference Example 2 were incubated according to procedures comparable to those in Application Example 1 (F-12 was used as a medium).

The determination of PreS-HBsAg was executed based on the ELISA method.

Table II

Sodium butyrate (mM)	PreS-HBsAg generation (mg/mL·day)
0	75
5	170

Application Example 3

The TPA generative transformed strains obtained in Reference Example 3 were incubated according to procedures comparable to those in Application Example 1. Either F-12 or Dulbecco's modified Eagle medium (DME) inclusive of 35 μg/mL of proline was used as a medium. The determination of t-Pa was executed based on the fibrin flat panel method. UK was used as a standard sample, whereas the activity was converted into UK international units.

Table III

Medium	Sodium butyrate (mM)	t-PA generation (U/mL·day)
F-12	0	5.3
	5	7.0
Pro + DME	0	11.4
	5	15.0

Experimental Example 1 (concentration)

200,000 cells of the ProUK generative transformed strain (C-68-53 strain) obtained in Reference Example 1 were implanted into 4 mL of an F-12 medium inclusive of 10 v/v% of bovine fetal serum (60 mm dish) and then incubated at 37°C for 3 days, as a result of which the cell count reached 2.8 $\times 10^6$. After the medium had then been removed, the incubation dish was washed with 4 mL of F-12, and subsequently, 4 mL of an F-12 medium inclusive of sodium butyrate (eventual concentration: 0 ~ 20 mM) was dispensed into each dish. After the cells had been incubated over a 24-hour period, the UK activity of the incubation supernatant was measured based on the fibrin flat panel method. Moreover, after the sample for measuring the UK activity had been collected, a trypsin treatment was performed, and after the cells had been peeled off

the incubation dish, the number of cells was counted by using a Coulter counter.

Table IV

Sodium butyrate (mM)	ProUK generation (U/mL·day)	Cell count (x 10^6)
0	56	4.3
1.25	75	4.4
2.5	94	3.6
5	106	2.8
10	114	2.3
20	95	2.0

Experimental Example 2 (type)

200,000 cells of the ProUK generative transformed strain (C-68-53 strain) obtained in Reference Example 1 were implanted into 4 mL of an F-12 medium inclusive of 10 v/v% of bovine fetal serum (60 mm dish) and then incubated at 37°C for 3 days, as a result of which the cell count reached 2.8 x10^6. After the medium had then been removed, the incubation dish was washed with 4 mL of F-12, and subsequently, 4 mL of an F-12 medium inclusive of any of various additives was dispensed into each dish. After the cells had been incubated at 37°C over a 24-hour period, the UK activity

of the incubation supernatant was measured based on the fibrin flat panel method.

Table V

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Additives	Concentration	ProUK generation ratio
None	-	100
Sodium chloride	5 mM	118
Sodium butyrate	5 mM	246
Calcitonin	100 ng/mL	107
Mezerein	160 mM	113

Experimental Example 3 (incubation time)

200,000 cells of the ProUK generative transformed strain (C-68-53 strain) obtained in Reference Example 1 were implanted into 4 mL of an F-12 medium inclusive of 10 v/v% of bovine fetal serum (60 mm dish) and then incubated at 37°C for 3 days, as a result of which the cell count reached 2.8 $x10^6$. After the medium had then been removed, the incubation dish was washed with 4 mL of F-12, and subsequently, 4 mL of an F-12 medium inclusive of 5 mM sodium butyrate was dispensed into each dish. After the cells had been incubated at 37°C over a period of 6 ~ 24 hours, the UK activity of the incubation supernatant was measured based on the fibrin flat panel method. The effect of fortifying the generation of

ProUK by sodium butyrate over each period in a case where the activity of a case where incubation was carried out by using the F-12 medium alone was defined as a control was assessed in terms of percentages.

Table VI

Incubation time (hr)	ProUK generation ratio
6	113±16
12	137±7
18	210±25
24	227±20

The numerical values signify the means ± standard deviations (number of cases: 4).

Experimental Example 4

In order to investigate the activation mechanism of the generation of ProUK by sodium butyrate, an incubation supernatant and a cell extract liquid of a UK generative strain were prepared, and their UK activities and protein internalization ratios were measured.

660,000 cells of the ProUK generative transformed strain (C-68-53) obtained in Reference Example 1 were implanted into 10 mL

of an F-12 medium inclusive of 10 v/v% of bovine fetal serum (100 mm dish), incubated for 3 days, and then washed with 10 mL of F-12. 10 mL of either an F-12 medium or an F-12 medium inclusive of 5 mM sodium butyrate was then dispensed into each dish. After the cells had been incubated at 37°C over a 24-hour period, the UK activities and protein internalization ratios of the cell extract liquid and incubation supernatant were respectively measured based on the fibrin flat panel method and Lowry method.

Method for preparing a cell extract liquid: Cells abiding in a singular layer (number of cells:; approximately 5,000,000) were collected by means of a trypsin treatment, washed twice with 10 mL of an equitensive phosphate buffer, and suspended in 0.5 mL of an equitensive phosphate buffer, and after 0.5 mL of a 0.5 v/v% Triton X-100 solution had subsequently been added, the obtained mixture was agitated and then statically placed at 37°C over a 5-min. period, as a result of which the targeted sample was obtained.

Table VII

Sodium butyrate (mM)	Fraction	UK activity (U/mL·day)	Protein internalization ratio (μg/mL)	Specific activity (U/μg·day)

0	Extract	7.7	820	9.4×10^{-3}
0	Extract	8.4	880	9.5×10^{-3}
5	Extract	39	692	56×10^{-3}
5	Extract	39	708	55×10^{-3}
0	Supernatant	39	36	1.30
0	Supernatant	39	32	1.40
5	Supernatant	39	40	2.78
5	Supernatant	39	40	2.63

The results of Table VII on the extracted fractions prove that the syntheses of /6 heterologous proteins introduced into cells can selectively be accelerated by the sodium butyrate treatment.

Moreover, it is suggested that the butyric acid treatment is equally applicable to a case where a plasmid that codes non-secretive products is used.

Reference Example 1

The ProUK generative transformed strain can be prepared by the method disclosed in Japanese Patent Application Publication Kokai No. Sho 61[1986]-177987.

In other words, the strain in question is derived from CHO-K_1 cells into which neomycin-resistant genes and ProUK genes have been co-introduced (i.e., cotransfection).

(1): cDNA

The cDNA is prepared from mRNA, which has been extracted from human kidney-derived strain-formable cells capable of generating UK.

All the analyzed base sequences are zirconium below.

Base sequences and amino acid sequences of proUK structural genes, signal sequence, and non-coding region

配列：ｐｒｏＵＫの構造遺伝子、signal sequence

及びnon-coding region の塩基配列とアミノ酸配列

5'TCCACCTGTCCCCGCAGCGCCGGCTCGCGCCCTCCTGCCGCAGCCACCGAGCCGCCGTCTACCGCCCCGACCTCGCCACC

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-20                                             -10                                  -1
Met Arg Ala Leu Leu Ala Arg Leu Leu Leu Cys Val Leu Val Val Ser Asp Ser Lys Gly
ATG TGA GCC CTG CTG GCG CGC CTG CTT CTC TGC GTC CTG GTC GTG AGC GAC TCC AAA GGC

  1                                          10                                  20
Ser-Asn-Glu-Leu-His-Gln-Val-Pro-Ser-Asn-Cys-Asp-Cys-Leu-Asn-Gly-Gly-Thr-Cys-Val-
ATC AAT GAA CTT CAT CAA GTT CCA TCG AAC TGT GAC TGT CTA AAT GGA GGA ACA TGT GTG

 21                                         30                                  40
Ser-Asn-Lys-Tyr-Phe-Ser-Asn-Ile-His-Trp-Cys-Asn-Cys-Pro-Lys-Lys-Phe-Gly-Gly-Gln-
TCC AAC AAG TAC TTC TCC AAC ATT CAC TGG TGC AAC TGC CCA AAG AAA TTC GGA GGG CAG

 41                                         50                                  60
His-Cys-Glu-Ile-Asp-Lys-Ser-Lys-Thr-Cys-Tyr-Glu-Gly-Asn-Gly-His-Phe-Tyr-Arg-Gly-
CAC TGT GAA ATA GAT AAG TCA AAA ACC TGC TAT GAG GGG AAT GGT CAC TTT TAC CGA GGA

 61                                         70                                  80
Lys-Ala-Ser-Thr-Asp-Thr-Met-Gly-Arg-Pro-Cys-Leu-Pro-Trp-Asn-Ser-Ala-Thr-Val-Leu-
AAG GCC AGC ACT GAC ACC ATG GGC CGG CCC TGC CTG CCC TGG AAC TCT GCC ACT GTC CTT

 81                                         90                                 100
Gln-Gln-Thr-Tyr-His-Ala-His-Arg-Ser-Asp-Ala-Leu-Gln-Leu-Gly-Leu-Gly-Lys-His-Asn-
CAG CAA ACG TAC CAT GCC CAC AGA TCT GAT GCT CTT CAG CTG GGC CTG GGG AAA CAT AAT
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101                                 110                                 120
Tyr-Cys-Arg-Asn-Pro-Asp-Asn-Arg-Arg-Arg-Pro-Trp-Cys-Tyr-Val-Gln-Val-Gly-Leu-Lys-
TAC TGC AGG AAC CCA GAC AAC CGG AGG CGA CCC TGG TGC TAT GTG CAG GTG GGC CTA AAG

121                                 130                                 140
Pro-Leu-Val-Gln-Glu-Cys-Met-Val-His-Asp-Cys-Ala-Asp-Gly-Lys-Lys-Pro-Ser-Ser-Pro-
CCG CTT GTC CAA GAG TGC ATG GTG CAT GAC TGC GCA GAT GGA AAA AAG CCC TCC TCT CCT

141                                 150                                 160
Pro-Glu-Glu-Leu-Lys-Phe-Gln-Cys-Gly-Gln-Lys-Thr-Leu-Arg-Pro-Arg-Phe-Lys-Ile-Ile-
CCA GAA GAA TTA AAA TTT CAG TGT GGC CAA AAG ACT CTG AGG CCC CGC TTT AAG ATT ATT

161                                 170                                 180
Gly-Gly-Glu-Phe-Thr-Thr-Ile-Glu-Asn-Gln-Pro-Trp-Phe-Ala-Ala-Ile-Tyr-Arg-Arg-His-
GGG GGA GAA TTC ACC ACC ATC GAG AAC CAG CCC TGG TTT GCG GCC ATC TAC AGG AGG CAC

181                                 190                                 200
Arg-Gly-Gly-Ser-Val-Thr-Tyr-Val-Cys-Gly-Gly-Ser-Leu-Ile-Ser-Pro-Cys-Trp-Val-Ile-
CGG GGG GGC TCT GTC ACC TAC GTG TGT GGA GGC AGC CTC ATC AGC CCT TGC TGG GTG ATC

201                                 210                                 220
Ser-Ala-Thr-His-Cys-Phe-Ile-Asp-Tyr-Pro-Lys-Lys-Glu-Asp-Tyr-Ile-Val-Tyr-Leu-Gly-
AGC GCC ACA CAC TGC TTC ATT GAT TAC CCA AAG AAG GAG GAC TAC ATC GTC TAC CTG GGT

221                                 230                                 240
Arg-Ser-Arg-Leu-Asn-Ser-Asn-Thr-Gln-Gly-Glu-Met-Lys-Phe-Glu-Val-Glu-Asn-Leu-Ile-
CGC TCA AGG CTT AAC TCC AAC ACG CAA GGG GAG ATG AAG TTT GAG GTG GAA AAC CTC ATC

241                                 250                                 260
Leu-His-Lys-Asp-Tyr-Ser-Ala-Asp-Thr-Leu-Ala-His-His-Asn-Asp-Ile-Ala-Leu-Leu-Lys-
CTA CAC AAG GAC TAC AGC GCT GAC ACG CTT GCT CAC CAC AAC GAC ATT GCC TTG CTG AAG
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(次頁に続く)

K33 is prepared as a plasmide inclusive of all base sequences of ProUK based on /7
an ordinary method.

(2): Insertion of a vector into a urokinase DNA sequence

After the pUK33 obtained in (1) (inclusive of urokinase cDNA with a total length of approximately 2.2 Kbp) had been partially digested with Pst I, a 1.7 Kbp fragment was isolated and then inserted into pSV-G_1. In the course of this reaction, the temperature of a liquid reaction mixture (pUK33: 100 µg; 10 x Pst I buffer: 50 µL; Pst I: 10 µL (60 U); balance of dH_2O for achieving a total of 500 µL) was maintained at 37°C, and after approximately 160 µL each had been sampled 10, 15, and 20 min. after the beginning of the reaction, it was thermally treated at 65°C over a 5-min. period, and after the respective reaction solutions had been mixed with one another, the obtained mixture was subjected to a 1% agarose gel electroforesis, as a result of which approximately 10 µg of a 1.7 Kbp fragment was recovered.. A Kpn I linker was added by substituting, by means of a T_4-DNA polymerase treatment, the adhered terminal with a smooth terminal by using approximately 5 µg of the recovered 1.7 Kbp fragment. Subsequently, [the obtained product was] linked to a pSV-G_1 digested with Kpn I for transforming *Escherichia coli* RB 101, as a result of which pSV-G_1-proUK (Figure 1) was obtained.

Inserted into the pSV-G_1-proUK on the lower stream side of the mRNA transfer adjustment realm of SV-40 were a proUK cDNA 5'-

non-coding region, a signal sequence coding region, a proUK coding region, and a 3'-non-coding region, whereas human ProUK can be generated by introducing the DNA into an appropriate recipient cell.

(3): Preparation of a plasmid DNA inclusive of a dominant selection marker

pSV-G_1-Neo^r, namely a plasmid used as a dominant selection marker in the context of transforming incubated cells by using the aforementioned pSV-G_1, was prepared according to the following procedures.

After a plasmid pNEO in which neomycin-resistant genes derived from Tn5 had been cloned (Southern, P. J. and P. Berg, *J. Molec. and Applied Genet.*, 1, 327 ~ 341 (1982)) had been cut by using Ba?? I and Hind III, they were precipitated in ethanol, and DNA fragments were then recovered. The adhered terminal of the recovered DNA was altered into a smooth terminal by using *E. coli* DNA-polymerase I, Klenow fragments in the presence of 4dNTP. The 1.5 Kbp DNA fragment was separated and recovered by means of agarose gel electroforesis, and subsequently, a Kpn I linker was added to its terminal according to the aforementioned procedures.

Upon the completion of the addition of the Kpn I linker, the DNA fragment was thoroughly digested by using Kpn 16 U, as a result of which the targeted DNA fragment of approximately 1.5 Kbp, to which the linker had been added, was recovered.

15 ng of the DNA fragment thus recovered was linked to 20 ng of a pSV-G_1 which had been cut with Kpn I according to procedures similar to the aforementioned ones, and the objective plasmid pSV-G_1-Neo^r was obtained as a result of the transformation of *Escherichia coli* HB101 (Figure 2).

(4): Preparation of a recipient cell

Chinese hamster ovary cells [CHO-K_1, ATCC CCL61 (purchased from Flow-Labo, Inc.)] were used as recipient cells, and said cells were incubated within a Hamm's F-12 medium (manufactured by Gibco Co.) inclusive of 10 v/v% of bovine fetal serum (manufactured by Boehringer Manheim Co.). The cells were intergenerationally maintained within a Falcon 3023 flask. Ones abiding in a single layer 2 days after the inoculation were used as cells to be transformed. After the cells had been collected by means of a trypsin treatment, they were suspended in the aforementioned incubation liquid and then implanted into a Falcon 3003 (100 mm) incubation laboratory dish at a density of 5×10^7 cells/10 mL. After the cells had been incubated within a CO_2 incubator (5% CO_2/95% air) over a 24-hour period, they were subjected to transformation. At this time, the cell count per laboratory dish was 7.0×10^7 cells.

(5): Transformation

1 mL of a microscopic DNA-$CaPO_4$ precipitate liquid inclusive of 2.0 μg/mL (in terms of DNA content) of a plasmid DNA [pSV-G_1-

proUK & pSV-G_1-Neo^r (mixing ratio: 100 : 1)] prepared according to the method of Osada, et al. and, as a carrier DNA, 20 µg/mL of a salmon semen DNA (PL-Biochemical) was added to each of the laboratory dishes prepared in (4). Each laboratory dish was mobilized crosswise and then placed statically placed, and after the microscopic DNA-$CaPO_4$ precipitate liquid had become adsorbed onto the cells, the incubation was resumed within the incubator. 18 hours later, the medium was exchanged, and after the incubation had been further continued for at least 48 hours, the selection of transformed cells was initialized.

(6): Selection of transformed cells

Transfected cells were selected by incubating cells within a medium inclusive of G-418 /8
(400 µg/mL) according to the method by Southern, et al. (*J. Molec. and Applied Genet.*, 1, 327 ~ 341, 1982) by using, as a dominant marker, a neomycin-resistant gene derived from Tn5, which is known to confer G-418 resistance onto eucaryotic cells (i.e., above-mentioned pSV-G_1-$Neo^?$). Incidentally, one manufactured by Gibco Co. (Geneticin, Gibco, lot No. 75 K6043 & 74N8040) was used as G-418.

Media were exchanged every 4 days, and after G-418-resistant colonies had been progressively cloned and grown, 83 clones were obtained on the tenth day. The generation of urokinase in the obtained clones was investigated by using a human urokinase RPHA reagent (manufactured by Midori Jujisha Co.) and based on the

fibrin flat panel method. As a result of primary screening with the RPHA reagent, approximately 25% of the G-418-resistant strains were acknowledged to be flocculated, from which the generation of a human urokinase-like substance was estimated.

In a case where the plasminogen activator activity of the 15 flocculated strains was examined by the fibrin flat panel method, activity was acknowledged within the incubation supernatant, and furthermore, this activity was neutralized by an anti-human urokinase antibody which had been purified within a urokinase antigen column. A transformed C-68 strain with a presumably high generation yield of the urokinase-like substance obtained as a result of screening was selected and then cloned by means of low-density incubation, as a result of which two strains of C-68-53 and C-68-61 were obtained.

The properties of the synthesized and secreted human UK-like proteins were investigated by using incubation supernatants of these clones. Incidentally, both clones of C-68-53 and C-68-61 secreted, into the medium, human UK-like proteins in stable and consistent fashions for more than 100 days. Moreover, the generation of a pro-UK to which a gluton saccharide chain with a molecular weight of 54,000 had been added was verified within the transformed cells.

Reference Example 2

$CHO-K_1$ cells wherein preS-HBsAg genes had been introduced and connected on the lower stream side of the SV 40 enhancer and

initial promotor were used as PreS-HBsAg generative transformed strains.

(1): Preparation of pSV-3rd reconstituted DNA (Figure 3)

A 1.2 Kbp fragment inclusive of the 3rd ATG of the PreS region through the HBsAg gene region (3rd PreS-HBsAg) was cut and removed from the plasmid pPS411, wherein an entire PreS-HBsAg structured gene had been inserted into the Hind III segment of pBR322, by using Mst II & Hind III, and a plasmid pSV-3rd which had been inserted, along the positive direction, into the EcoR I segment of the animal cell expression vector $pSVG_2$-Eco was prepared.

10 μg of pPS411 was reacted with 12 U of Mst II and 12 U of Hind III at 37°C for 5 hours, extracted twice with phenol, and then precipitated in ethanol, as a result of which a DNA was recovered. This DNA was reacted with 10 U of a DNA polymerase I Klenow fragment at room temperature for 30 min. in the presence of a 40 mM tris-hydrochloric acid buffer (pH: 7.2), 8 mM magnesium sulfate, 80 μM dithiothreitol, and 2 mM dNTP for converting its adhered terminal into a smooth terminal, and after it had then been reacted with 1 U of a bovine small intestine-derived alkali phosphatase within a 50 mM tris-hydrochloric acid buffer (pH: 8.2) at 37°C for 1 hour for dephosphating its 5' terminal, it was extracted twice with chloroform and phenol and then precipitated in ethanol, as a result of which a DNA was recovered. 4 μg of this DNA was reacted with 5.6 U of T4DNA ligase at 16°C overnight

in the presence of a 66 mM tris-hydrochloric acid buffer (pH: 7.6), 6.6 mM magnesium chloride, 10 μM dithiothreitol, 1.0 mM ATP, and 2 μg EcoRI linker (pGGAATTCC), as a result of which the EcoRI linker became connected. This DNA was precipitated in ethanol and then recovered, and after it had subsequently been reacted with 50 U of EcoR I at 37°C for 5 hours, it was subjected to a 0.8% low-melting-point agarose gel electroforesis, and after a 1.2 Kbp fragment had been cut and removed from the gel, it was extracted twice with phenol, and a DNA was recovered as a result of ethanol precipitation.

The PreS-HBsAg 1.2 Kbp fragment to which the EcoR I linker had been connected was inserted into the EcoR I segment of the animal cell expression vector $pSVG_2$-Eco in possession of an SV40 enhancer, promotor, splicing junction, and polyadenylation signal. 3 μg of $pSVG_2$-Eco was reacted with 10 U of EcoR I at 37°C for 6 hours and then reacted with 20 U of a bovine small intestine-derived alkali phosphatase within a 50 mM tris-hydrochloric acid (pH: 8.2) buffer at 37°C for 1 hour for dephosphating its 5' terminal, and subsequently, it was subjected to a 0.8% low-

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melting-point agarose gel electroforesis. After the electroforesis, the fragment was cut and removed, extracted twice with phenol, and a DNA was recovered as a result of ethanol precipitation. 1 μg of EcoR I digested $pSVG_2$-Eco and 0.5 μg of a PreS-HBsAg 1.2 Kbp fragment were reacted with 8.4 U of T4DNA ligase at 16°C overnight within a reaction solution of a 66 mM

tris-hydrochloric acid buffer (pH: 7.6), 6.6 mM magnesium chloride, 10 mM dithiothreitol, and 1.0 mM ATP, as a result of which the goal of transformation into *Escherichia coli* HB101 was achieved, and an ampicillin-resistant transformed strain was obtained. Severance patterns of EcoRI, Ba?H I, & Sal I + Xba I within the transformed strain were analyzed, as a result of which a pSV-3rd into which a pre S-HBsAg fragment had been inserted along the positive direction thereof was obtained.

(2): Transformation of animal cell.

The transformation of the CHO-K_1 cell is executed based on the calcium phosphate precipitation method. 2.5 mL of a HEPES buffer with a doubled concentration (2 x HBS) [HEPES(N-(2-hydroxyethyl)piperazine-N'-2-ethanesulfonic acid): 10 g/L; NaCl: 6 g/L; adjusted at a pH of 7.1), 50 μL of an aqueous solution of 70 mM NaH_2PO_4 and 70 mM Na_2HPO_4, 100 μL of a 1 μg/mL salmon semen DNA, and 100 μL of the reconstituted DNA [pSV-3rd: 10 μg; pSV-G_1-Neo^r (Figure 2): 0.1 μg)] were filled into a tube, and after the total volume had been adjusted at 4.7 mL with sterilized water, the solution B was obtained. 300 μL of the solution A (2 M $CaCl_2$) was added to this solution B. The solution A was slowly added dropwise into the solution B, which was being agitated in an air-pumped state. Upon the completion of the addition of the solution A, air was continuously pumped for 1 ~ 2 min., as a result of a homogeneous precipitate was formed, and it was then statically placed for 10 min. The CHO-K_1 cells were distributed within a 100

mm laboratory dish at a density of 5 x $10^?$ on the day before transfection and then incubated within a CO_2 incubator for 24 hours. A Hamm's F-12 medium inclusive of 10 v/v% of bovine fetal serum was used as an incubation liquid. 1 mL of the microscopic precipitate liquid was dropped onto this incubation laboratory dish and then diffused homogeneously. After the DNA-calcium phosphate microscopic precipitate had been adsorbed onto the cells as a result of a 10-min. static unattendance period, the cells were further incubated within the CO_2 incubated. The medium was exchanged 18 hours after the transfection, and [the exchanged medium was] further exchanged with a medium inclusive of G-418 48 hours later. The concentration of G-418 was 400 μg/mL. After the colony grown within the medium inclusive of G-418 had been separated by the cylinder method, G-418-resistant transformed cells were obtained.

(3): Generation of Pre S-HBsAg

The HBsAg activity of the aforementioned G-418-resistant cells within the incubation liquid was investigated based on the RPHA method (Anti-Hepcel, manufactured by Midori Juji Co.) and EIA method (Authzyme II, manufactured by Dynapot Co.). HBsAg positive strains are obtained in high frequency from the G-418-resistant strains. The polyalbumin (pHSA) coupling activity, furthermore, was investigated based on the PHA method for polyalbumin (pHSA) (Lenkey, et al., *Immunol. Methods*, 16, 23 ~ 30, 1977), as a result of which the pHSA coupling activity was acknowledged in the HBsAg

positive cell supernatant, and the expression of a Pre S region was verified. This supernatant was then purified based on the cesium chloride density gradient centrifugal separation method. In a case where this purified sample was analyzed by means of SDS PAGE, three bands with molecular weights of approximately 35,000, approximately 27,000, and approximately 24,000, respectively, were verified. It was thus verified that Pre S-HBsAg peptide had been generated from the transformed cells. Incidentally, in a case where the Pre S-HBsAg particles obtained in the present invention were observed by using an electron microscope, they were determined to be spherical particles with diameters of approximately 22 nm.

Reference Example 3

CHO-K_1 cells wherein t-PA genes had been introduced and connected on the lower stream side of the SV-40 enhancer and initial promotor were used as a t-PA generative transformed strain.

(1): Building of an expression vector

A fragment inclusive of all coding regions of t-PA was cut and removed from a plasmid pTPA-2 in possession of a t-Pac DNA derived from a melanoma cell G-361 strain and then inserted in-between expression adjustment regions of the animal cell expression vector pSV-G_2, as a result of which the t-PA expression vector pDX-2 was built.

(1-i): Preparation of pXK-2 (Figure 4)

Plasmid pTPA-2 was digested with Hind III and Xmn I, and after it had then been subjected to agarose gel electroforesis, a 2.9 Kb DNA band inclusive of t-PAc DNA was cut and removed, and the DNA was recovered by means of electroelution.

After both terminals of this 2.9 Kb Hind III-Xmn I fragment had been filled in, a Kpn I linker was added. The fragment thus coupled with the Kpn I linker was further digested with /10
Kpn I, and after a band in the vicinity of 2.9 Kb had been cut and removed by means of agarose gel electroforesis, a DNA was recovered by means of electroelution.

The animal cell expression vector pSV-G_2, on the other hand, was digested with Kpn I and then treated with CIP (bovine small intestine-derived alkali phosphatase), as a result of which the phosphoric acid group at the 5' terminal was removed. Next, this 3.2 Kb pSV-G_2-Kpn I fragment and the previously prepared fragment inclusive of 2.9 Kb t-PAc DNA were mutually linked. The *Escherichia coli* HB101 was transformed by using the solution obtained upon the completion of the reaction.

The targeted plasmid pXK-2 was selected from the plasmid DNA extracted from the transformed matter.

(1-ii): Preparation of pDD-2 plasmid (Figure 5)

Plasmid pTPA-2 was digested with Bbe I and Hind III, and after this DNA had been subjected to agarose gel electroforesis, a

DNA band of 1.1 Kb inclusive of a G·C tail and a portion of the t-PAc DNA was cut and removed from the obtained gel, and the DNA was then recovered by means of electroelution. After this Hind III-Bbe I fragment had been digested with Dde I, the protuberant terminal on the 5' side was then filled in, and after a 270 bp fragment band had subsequently been cut and removed from a polyacrylamide gel, the DNA was then recovered by means of electroelution. Kpn I linkers were added to both terminals of the 270 bp Dde I fragment from which the G·C tail had been removed. After this fragment had been temporarily digested with Kpn I, the DNA fragment derived from the linker DNA was removed by means of polyacrylamide gel electroforesis. After the electroforesis, a band in the vicinity of 270 pb was cut and removed from the gel, and the DNA was then recovered by means of electroelution.

The Kpn I-treated pSV-G_2 prepared in (1-i) and the previously prepared 270 bp Kpn I fragment were then mutually linked. The *Escherichia coli* DH1 was transformed by using the solution obtained upon the completion of the reaction.

The targeted plasmid pDD-2 was selected from the plasmid DNA extracted from the transformed matter.

(i-iii): Preparation of pDX-2 plasmid (Figure 6)

The plasmid pXK-2 prepared in (1-i) was digested with Ban III (isoschizomer of Cla I) and then partially digested further with BgI II, and after a BgI II segment positioned in the vicinity of the N terminal of t-PA alone had been severed, a DNA band of a 4.8

Kb Ban III-BgI II fragment was cut and removed from the gel by means of agarose gel electroforesis, and after the DNA had then been recovered by means of electroelution, the obtained fragment was treated with BAP (bacterially derived alkali phosphatase).

The pDD-2 prepared in (1-ii), on the other hand, was digested by using Ban III and BgI II. After it had then been subjected to agarose gel [electroforesis?], a 730 bp DNA band inclusive of an SV 40 initial promotor was cut and removed from the gel, and the DNA was then recovered by means of electroelution. This 730 bp Ban III-BgI II fragment and the previously prepared 4.8 Kb Ban III-BgI II fragment were mutually linked, and the *Escherichia coli* DH1 was transformed by using this reaction solution.

The targeted plasmid pDX-2 was selected from the plasmid DNA extracted from the transformed matter.

(2): Preparation of a t-PA generative transformed strain

CHO-K_1 cells were, together with pSV-G_1-Neo^r, cotransfected based on the DNA-calcium phosphate precipitation method by using the plasmid pDX-2 obtained in (1) according to procedures comparable to those in Reference Example 1 or 2.

The introduction efficiency was approximately 1.8×10^{-4} ~ 4.6×10^{-4} colonies/μg DNA/dish.

The targeted t-PA generative transformed strain (R-72-3) was selected from the obtained product.

4. Brief explanation of the figures

Figure 1 shows the procedures for building pSV-G_1 proUK (both supported) from pUK33 (cDNA of proUK supported) and pSV-G_1 (SV 40 initial promotor supported).

Figure 2 shows the procedures for building pSV-G_1Neo^r (both supported) from pNEO (neomycin-resistant gene supported) and pSV-G_1 (SV 40 initial promotor supported).

Figure 3 shows the procedures for building pSV-3rd (both supported) from pPS411 (cDNA of preS-HBsAg supported) and pSV-G_2-Eco (SV 40 initial promotor supported).

Figure 4 shows the procedures for building pXK-2 from pTPA-2 (cDNA of t-PA supported) and pSV-G_2 (SV 40 initial promotor supported). /11

Figure 5 shows the procedures for building pDD-2 from pTPA-2 and pSV-G_2.

Figure 6 shows the procedures for building pDX-2 (both cDNA of TPA and SV 40 initial promotor supported) from pXK-2 and pDD-2.

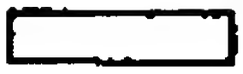
In the figures, the notations denote the following:


: SV 40 initial enhancer;


: SV 40 initial promotor;


: Neomycin-resistant gene;


: Exo genous DNA;


SV 40 polyadenylation signal;

Ap^r: Ampicillin-resistant;

Tc^r: Tetracycline-resistant;

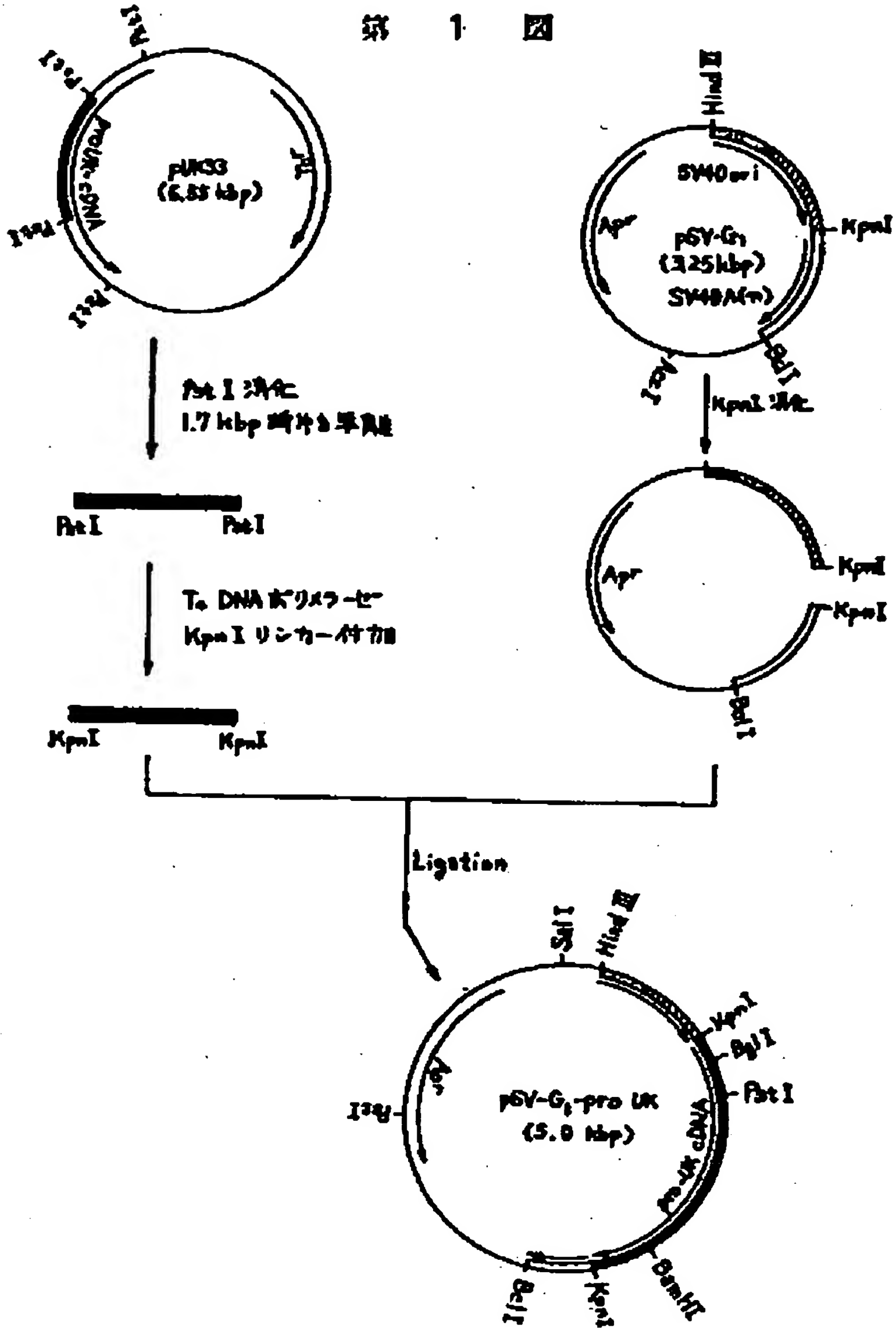
-: pBR332-derived.

Moreover, the limiting enzyme segments are expressed as follows: Ac: Acc I; B: BamH I; Ba: Bal I; Bb: Bbe I; C: Cla I; D: Dde I; GI: Bgl I; GII: Bgl II; H: Hind III; Hp: Hpa I; K: Kpn I; P: Pst I; Pv: Pvu II; S: Sal I; Sc: Sca I; Sa: Sph I; T: Tth IV; X: Xho I; Xm: Xmn I.

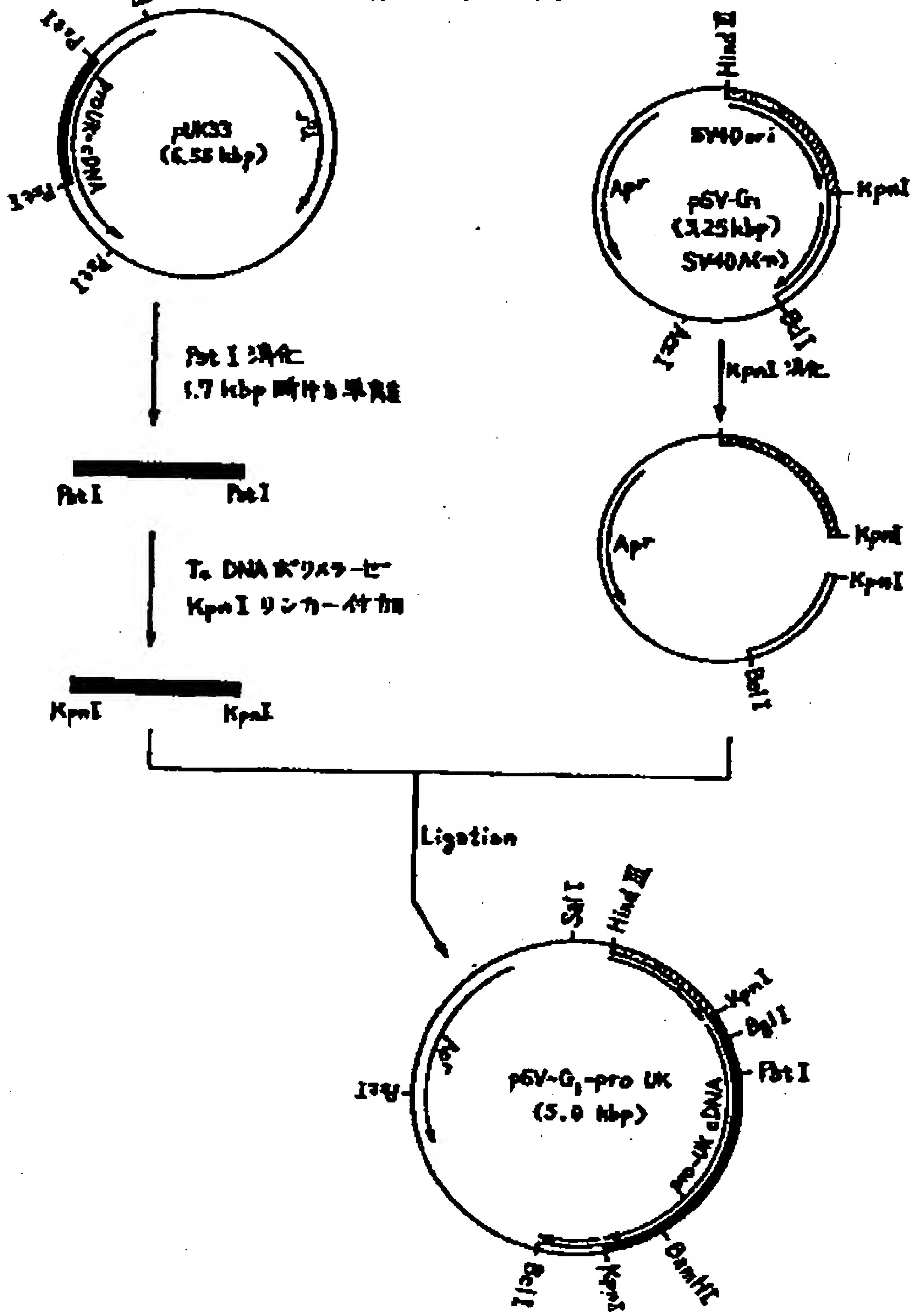
Agent: (8107) Kiyotaka Sasaki, patent attorney, and 3 others

Figure 1

第　1　図


pUK33
(6.85 kbp)
Pst I 消化
1.7 kbp 断片を単離
Pst I
Pst I
T4 DNA ポリメラーゼ
Kpn I リンカー付加
Kpn I
Kpn I
pSV-G1
(3.25 kbp)
Hind III
Kpn I
Kpn I 消化
Kpn I
Kpn I
Ligation
pSV-G1-pro UK
(5.0 kbp)
Sal I
Hind III
Kpn I
Bgl I
Pst I
BamHI
Kpn I
Bgl I

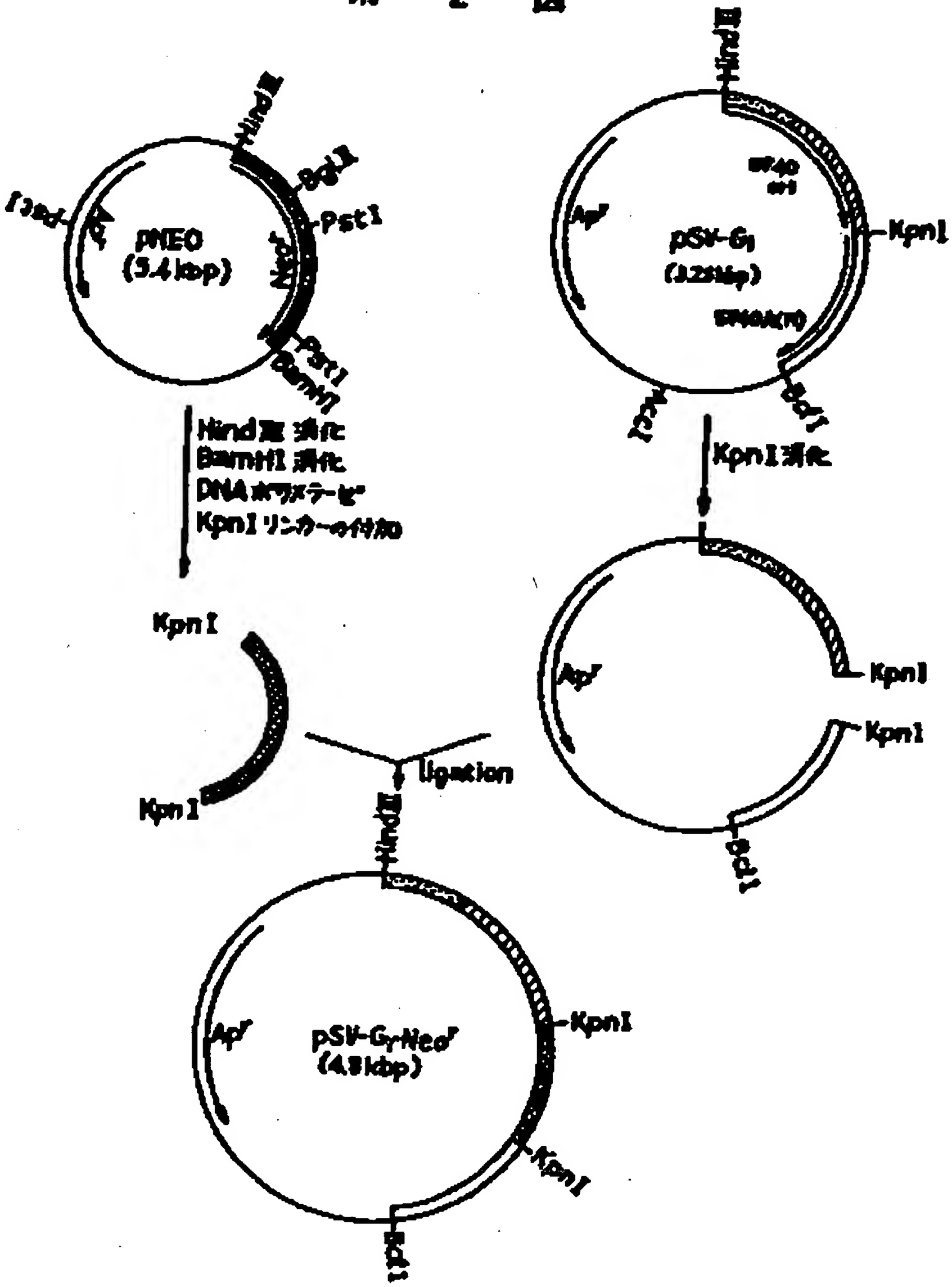
第 1 図

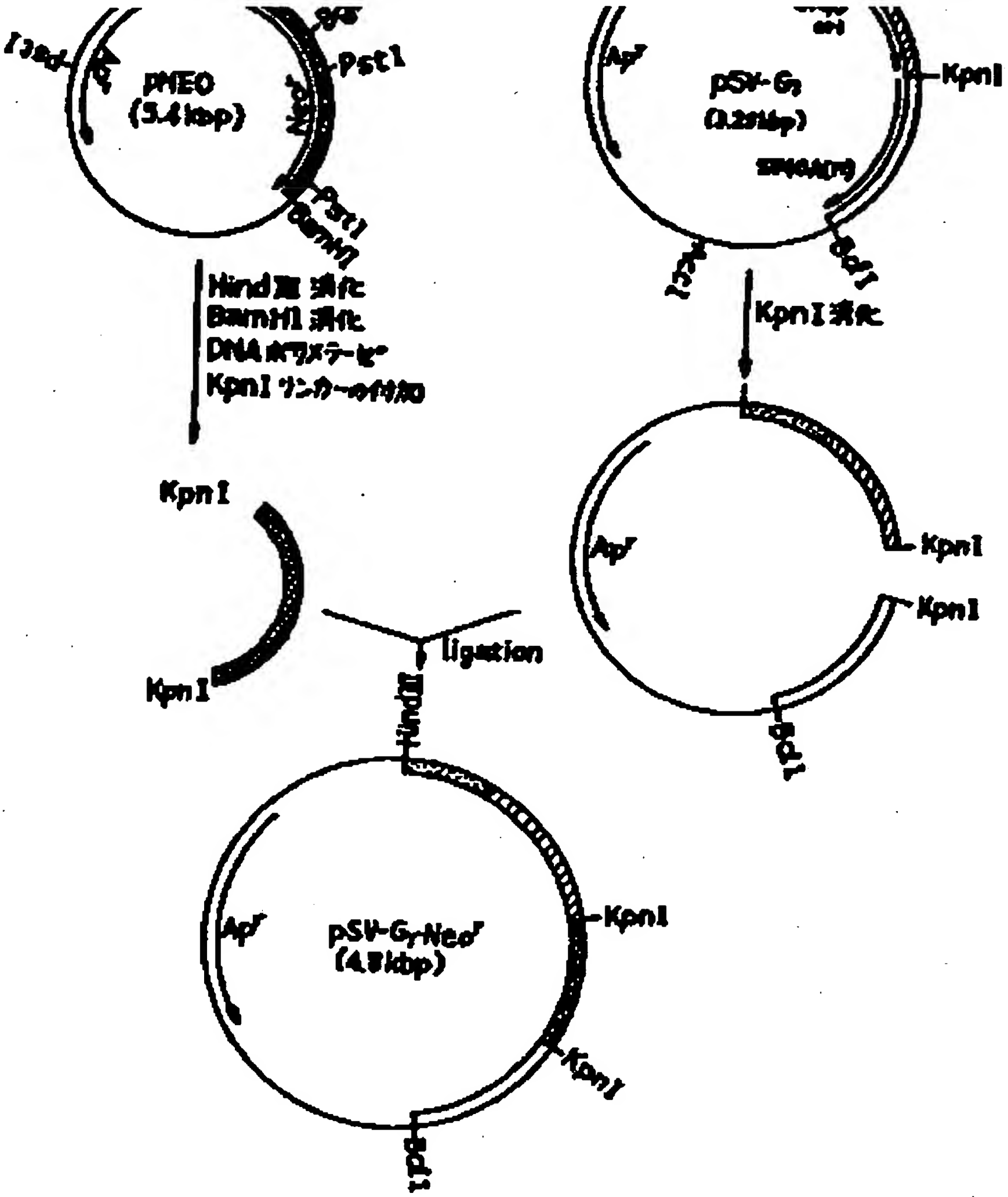

PstI
pUK33
(6.55 kbp)
pro UK-cDNA
Tcr
Hind III
SV40ori
Apr
pSV-G1
(3.25 kbp)
SV40A(n)
KpnI
BglI
PstI 消化
1.7 kbp 断片を単離
PstI
T4 DNA ポリメラーゼ
KpnI リンカー付加
KpnI
KpnI 消化
Ligation
SalI
Hind III
BglII
pSV-G1-pro UK
(5.0 kbp)
pro-UK cDNA
BamHI

[(A): Pst I digestion; (B): Isolation of 1.7 kbp fragment; (C): T_4 DNA polymerase; (D): Kpn I linker addition; (E): Kpn I digestion]

Figure 2

第　2　図


pNEO
(5.4 kbp)
HindIII
SphI
PstI
PstI
BamHI
Ap
ori
HindIII 消化
BamHI 消化
DNA ポリメラーゼ
KpnI リンカーの付加
KpnI
KpnI
ligation
HindIII
pSV-GγNeo
(4.8 kbp)
Ap
KpnI
KpnI
pSV-Gγ
(3.2 kbp)
ori
Ap
KpnI
KpnI 消化
KpnI
KpnI
SphI


pMEO
(3.4kbp)
Pst1
Pst1
BamHI
HindIII 消化
BamHI 消化
DNA ポリメラーゼ
KpnI リンカーの付加
KpnI
KpnI
ligation
HindIII
pSV-G
(3.2kbp)
Ap
ori
KpnI
PstI
BamHI
KpnI 消化
Ap
KpnI
KpnI
PstI
pSV-G-Neo
(4.8kbp)
Ap
KpnI
KpnI
PstI

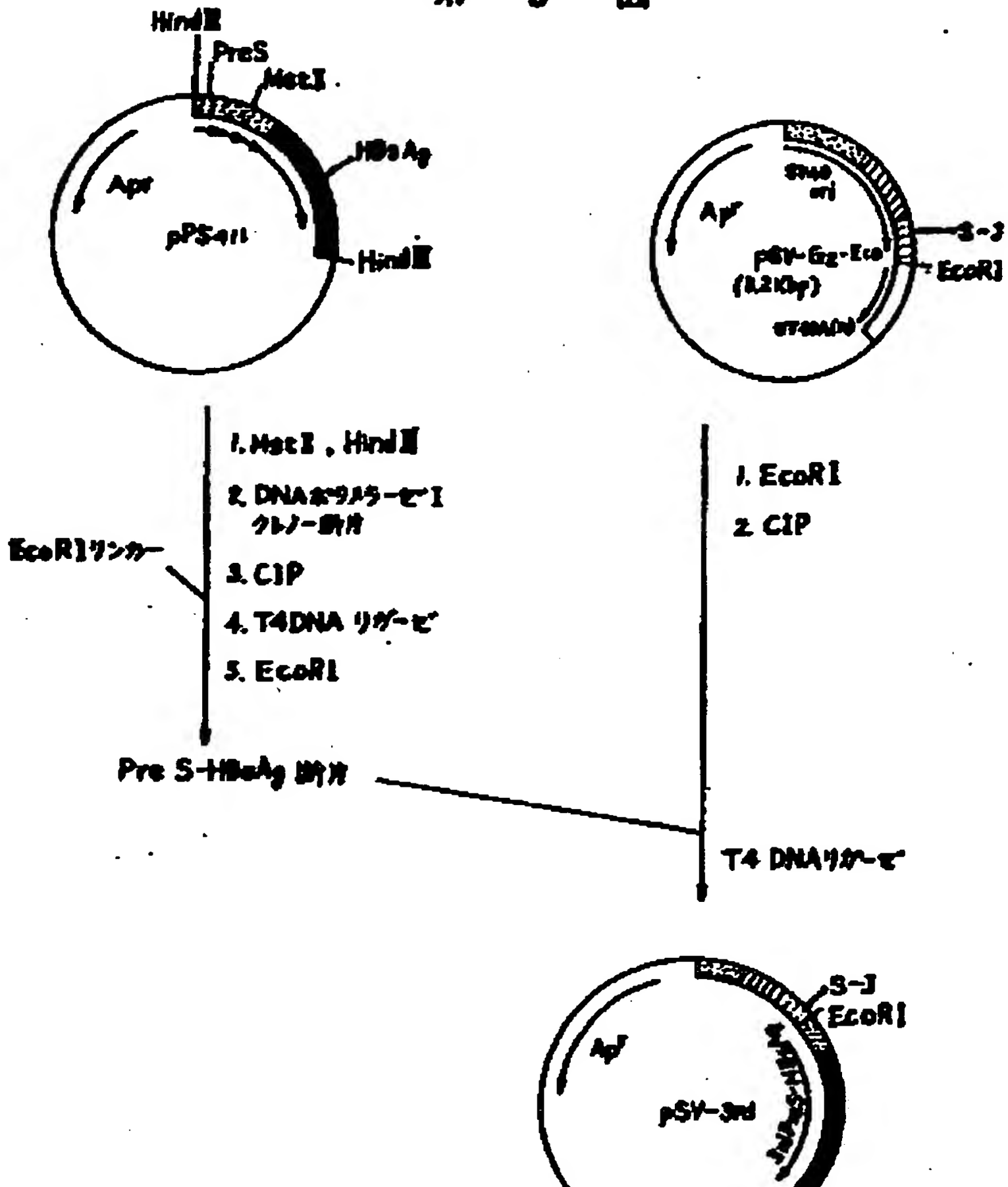
57—

[(A): Hind III digestion;; (B): Bam H1 digestion; (C): DNA polymerase; (E): KpnI linker addition; (E): Kpn I digestion]

Figure 3

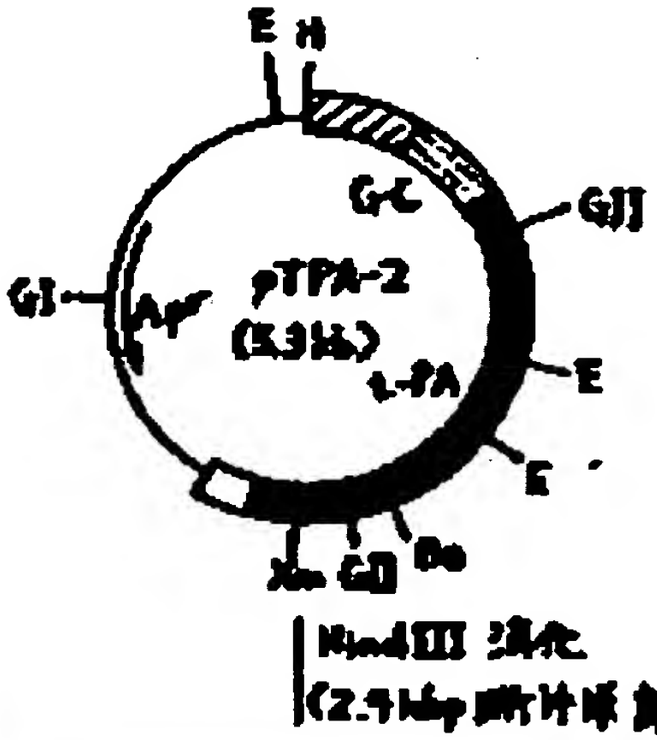
/12

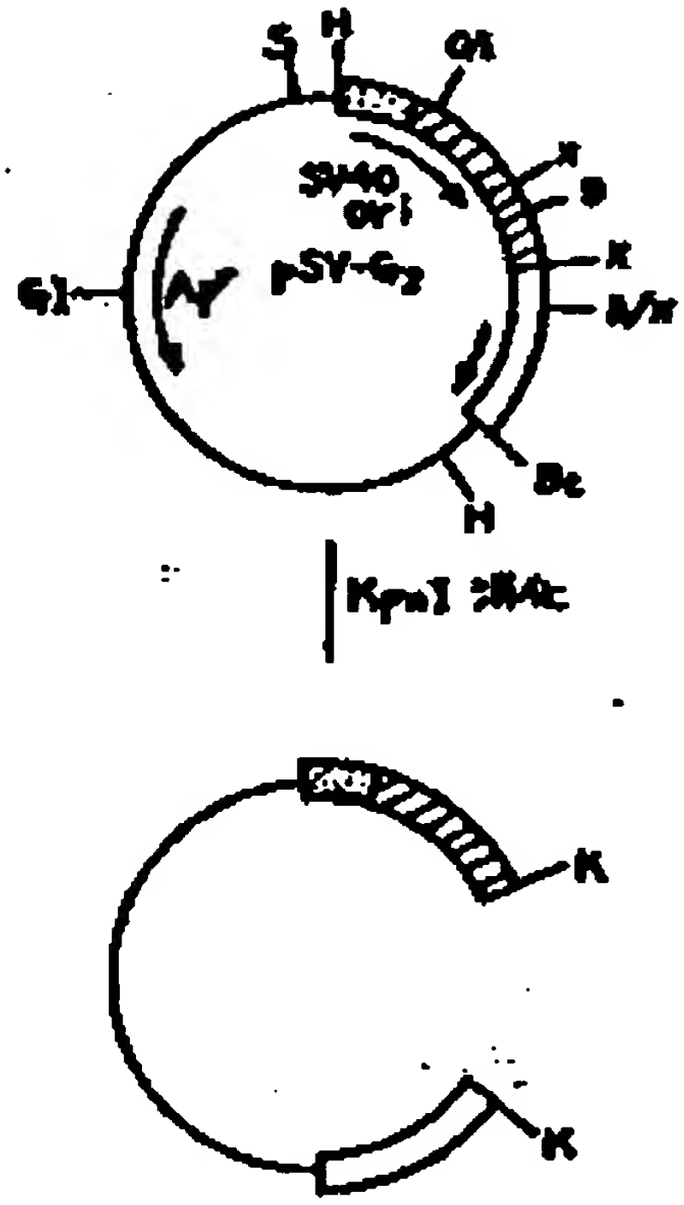
第 3 図

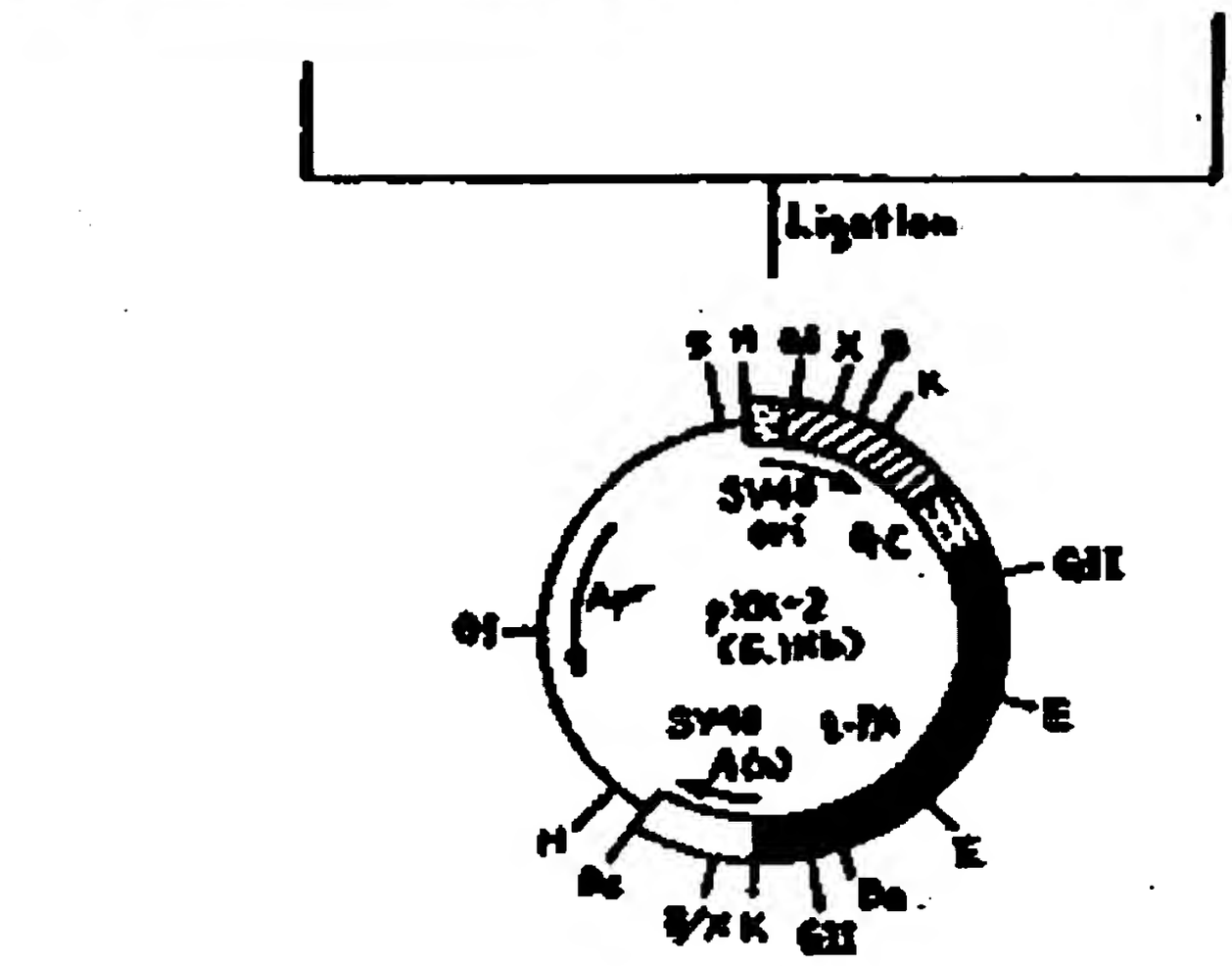


[(A): DNA polymerase I Klenow fragment; (B): T4 DNA ligase; (C): PreS-HBsAg fragment; (D): T4 DNA ligase; (E): EcoR I linker]

Figure 4

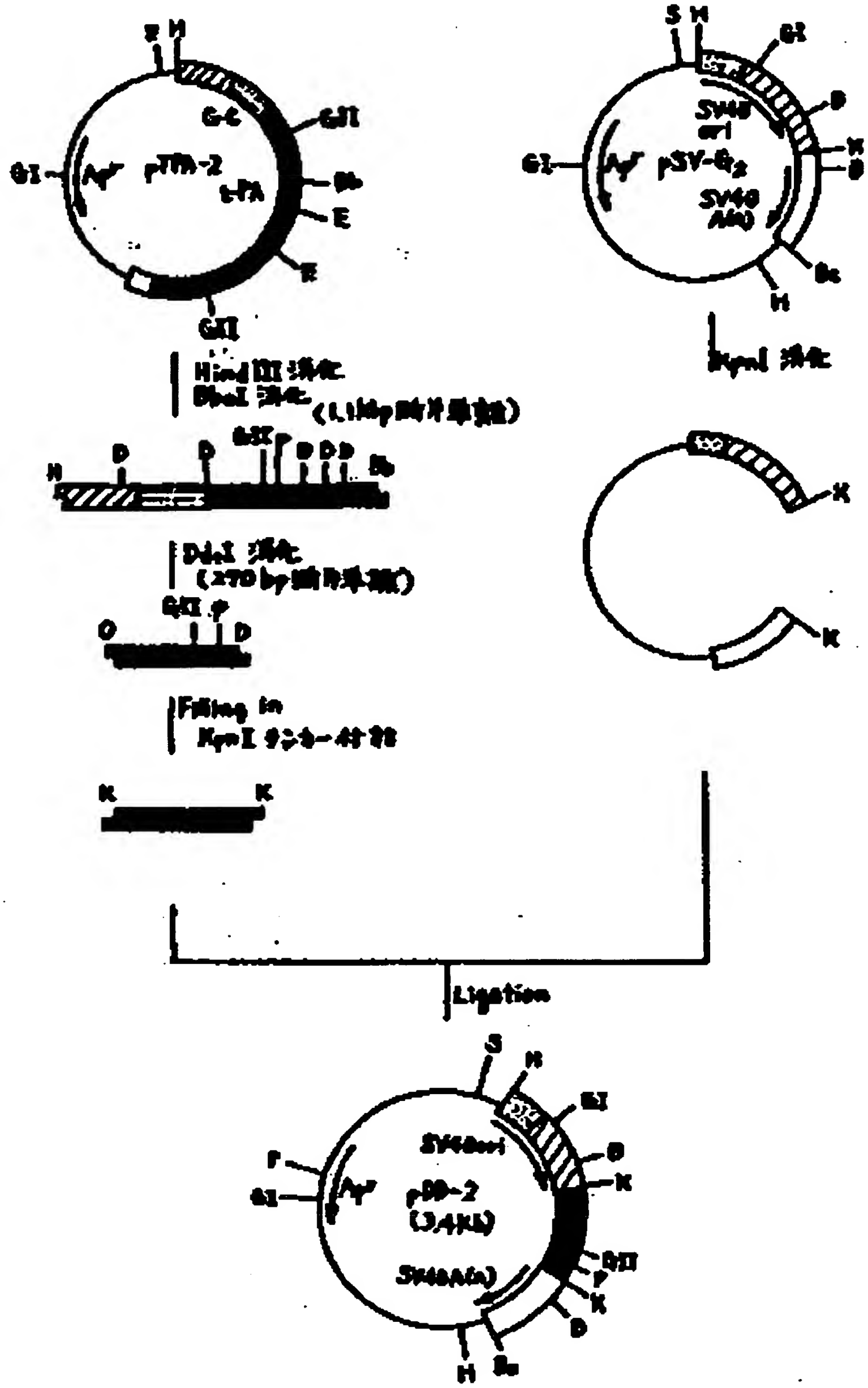

E H
G-C
G]]
G1
pTPA-2
(5.3kb)
t-PA
E
E
Xba G]]
Bam
HindIII 消化
(2.9kbp 断片単離)
H
K
Filling in
KpnI リンカー付加
K
K


S H
Ori
SV40
ori
pSV-G2
G1
Bc
H
KpnI 消化
K
K


Ligation
S H Bg X B
K
SV40
ori
G-C
G]]
pTPA-2
(5.3kb)
G1
SV40 t-PA
A(x)
H
E
Bc
Bam
B/X K
G]]

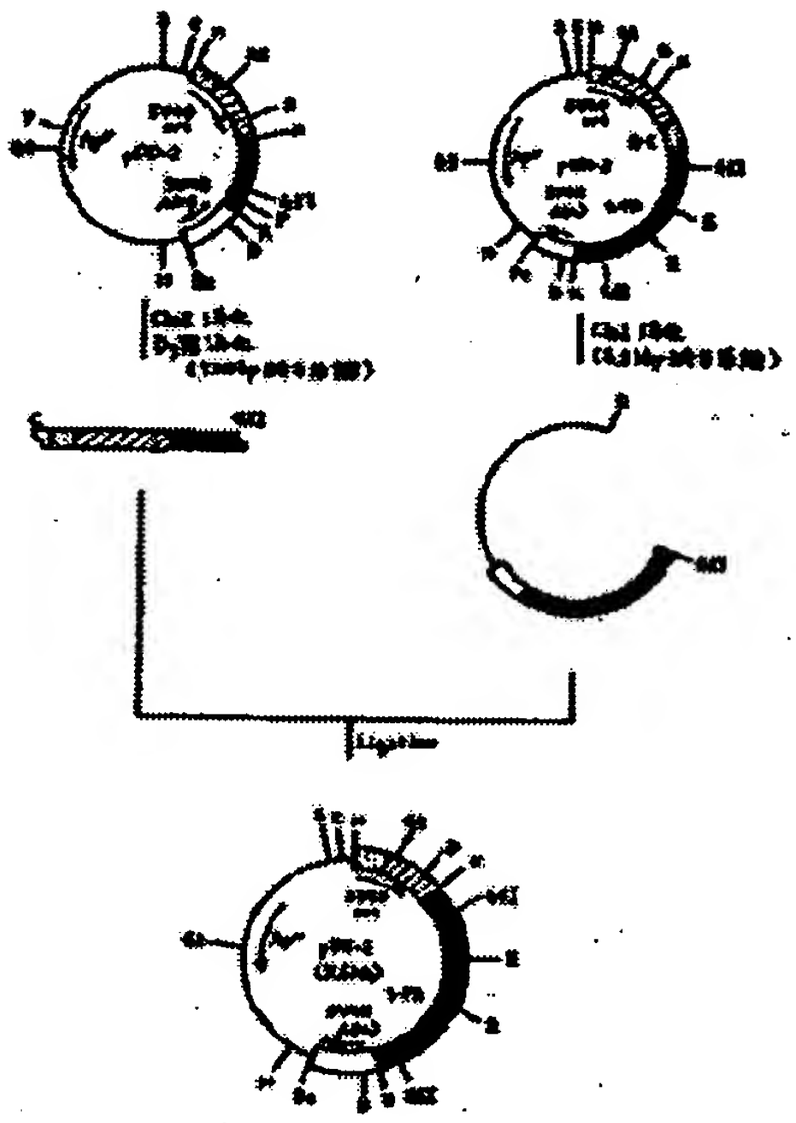
[(A): Hind III digestion; (B): (Isolation of 2.9 kbp fragment; (C): Kpn I linker addition; (D): Kpn I digestion]

Figure 5


Ligation

[(A): Hind III digestion; (B): Bbe I digestion (isolation of 1.1 kbp fragment); (C): Dde I digestion (isolation of 270 bp fragment); (D): Filling in, addition of Kpn I linker; (E): Kpn I digestion]

Figure 6



[(A): Cla I digestion; (B): Bg III digestion (isolation of TSObp fragment; (C): Cla I digestion (isolation of 4.? kbp fragment)]

Procedural amendment report

/13

May 1, Sho 62[1987]

Dear Chief Judge of the Patent Agency:

1. Display of the case

Japanese Patent Application Tokugan No. Sho 62[1987]-048935

2. Title of the invention

Method for fortifying the generation of heterologous proteins

3. Amending party

Relation to the case: Patent applicant

Name: Modiro Juji Co., Ltd.

4. Agent

Address: c/o Eiko Patent Office

No. 5, 2 ban, 3 chome, Kasumigaseki, Chiyoda-ku,

Tokyo-to, ZIP: 100 (29th floor, Kasumigaseki Bldg.)

P. O. Box #49, Kasumigaseki Bldg. Post Office

Phone: (581)-9601 (representative)

Name: (8107) Kiyotaka Sasaki, patent attorney, and 3 others

[Official seal]

5. Date of amendment order

Spontaneously issued

6. Number of inventions added as a result of the amendment

0

7. Target of amendment

"Detailed explanation of the invention" section of the specification

8. Contents of amendment

The "Detailed explanation of the invention" section of the specification is amended as follows.

[Stamped, "Patent Agency, May 1, Sho

62[1987], Second Application Division"]

1. "Co-transfection" in line 6, page 7 of the specification is amended as "cotransfection."

2. Table V on page 15 of the same is amended as follows:

Table V

Additives	Concentration	ProUK generation ratio
None	-	100
Sodium chloride	5 mM	118
Sodium butyrate	5 mM	246

3. The contents mentioned in the second line from the bottom to the last line of page 23 of the same are placed immediately after "*Escherichia coli* HB" on the second line from the bottom of the same page without a hard return.

4. The contents mentioned in lines 3 ~ 10 of page 27 of the same are placed immediately after "resistant gene" in line 2 of the same page without a hard return.

Procedural amendment report

August 3, Sho 62[1987]

Dear Chief Judge of the Patent Agency:

1. Display of the case

Japanese Patent Application Tokugan No. Sho 62[1987]-48935

2. Title of the invention

Method for fortifying the generation of heterologous proteins

3. Amending party

Relation to the case: Patent applicant

Name: Modiro Juji Co., Ltd.

4. Agent

Address: c/o Eiko Patent Office

No. 5, 2 ban, 3 chome, Kasumigaseki, Chiyoda-ku,

Tokyo-to, ZIP: 100 (29th floor, Kasumigaseki Bldg.)

P. O. Box #49, Kasumigaseki Bldg. Post Office

Phone: (581)-9601 (representative)

Name: (8107) Kiyotaka Sasaki, patent attorney, and 3 others

[Official seal]

5. Date of amendment order

Spontaneously issued

6. Number of inventions added as a result of the amendment

0

7. Target of amendment

Procedural amendment report form submitted on May 1, Sho 62[1987] on which the accurate title of the invention is listed

8. Contents of amendment

See the attached sheet

[Stamped, "Patent Agency, August 3, Sho 62[1987], Second Application Division"]

Procedural amendment report

May 1, Sho 62[1987]

Dear Chief Judge of the Patent Agency:

1. Display of the case

Japanese Patent Application Tokugan No. Sho 62[1987]-48935

2. Title of the invention

Method for fortifying the generation of heterologous proteins

3. Amending party

Relation to the case: Patent applicant

Name: Modiro Juji Co., Ltd.

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No. 5, 2 ban, 3 chome, Kasumigaseki, Chiyoda-ku,

Tokyo-to, ZIP: 100 (29th floor, Kasumigaseki Bldg.)

P. O. Box #49, Kasumigaseki Bldg. Post Office

Phone: (581)-9601 (representative)

Name: (8107) Kiyotaka Sasaki, patent attorney, and 3 others

[Official seal]

5. Date of amendment order

Spontaneously issued

6. Number of inventions added as a result of the amendment

0

7. Target of amendment

"Detailed explanation of the invention" section of the specification

8. Contents of amendment

The "Detailed explanation of the invention" section of the specification is amended as follows.